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Immunomediated and Ischemia-Independent Inflammation of Coronary Microvessels in Unstable Angina

Gian Gastone Neri Serneri, Maria Boddi, Pietro Amedeo Modesti, Ilaria Cecioni, Mirella Coppo, Maria Letizia Papa, Thomas Toscano, Antonio Marullo, Mario Chiavarelli

Abstract—This study investigated whether the myocardium is involved in the acute inflammatory reaction associated with bursts of unstable angina (UA). We looked for the presence of activated DR⁺ inflammatory cells and the expression patterns, localization, and immunostaining identification of genes for cytokines (IL-1 β , TNF- α , IL-6, and IFN- γ), MCP-1, and iNOS in the left ventricle biopsies from 2-vessel disease anginal patients, 24 with UA and 12 with stable angina (SA), who underwent coronary bypass surgery. Biopsy specimens from 6 patients with mitral stenosis who underwent valve replacement were examined as control hearts (CHs). Plasma levels of IL-2 soluble receptor (sIL-2R) were measured as a marker of systemic immune reaction. In CHs, DR⁺ cells were undetectable, and cytokine and iNOS mRNA expression were negligible. UA patients had higher sIL-2R levels than SA patients ($P<0.01$), and their biopsy specimens showed both numerous DR⁺ cells identified as lymphocytes, macrophages, endothelial cells, and elevated expression levels of cytokine and iNOS genes (from 2.4- to 6.1-fold vs SA; $P<0.01$). Cytokine and iNOS genes and proteins were localized in endothelial cells without involvement of myocytes. IL-1 β and MCP-1 mRNAs were nearly undetectable. No significant differences were found in the number of DR⁺ cells, levels of cytokine, and iNOS genes between potentially ischemic and nonischemic left ventricle areas. In SA specimens, DR⁺ cells were very rare and only mRNAs for TNF- α and iNOS genes were overexpressed versus CHs. These results indicated that an acute immunomediated inflammatory reaction, essentially involving coronary microvessels, is demonstrable in UA patients. (*Circ Res.* 2003;92:1359-1366.)

Key Words: unstable angina ■ coronary microvessels ■ myocardial inflammation

Patients with unstable angina (UA) class IIB and IIIB of Braunwald's classification have ischemia at rest or at very low workloads. The common cause of the transient reduction of coronary flow is considered to be a nonocclusive thrombus on a fissured or eroded atherosclerotic plaque that typically had caused only mild-to-moderate obstruction previously.¹ However, recent angioscopic studies performed on UA patients classified according to Braunwald's classification have shown that intracoronary thrombi are detectable only in a proportion of patients, ranging from 39% to 68%,²⁻⁶ and are more frequently found in patients with refractory angina. Because the sensitivity of percutaneous coronary angiography for detecting coronary lesions has been found to be superior to that of coronary angiography,⁷ it is reasonable to suppose that intracoronary thrombi remain undetected in at least 30% of class IIB and IIIB UA patients. Vasoconstriction at both the site of stenosis or thrombus and at the microvascular level may play a contributory role in reducing coronary blood flow.¹ In atherosclerosis a reduced endothelium-dependent relaxation has been demonstrated both at the level of epicardial coronary arteries^{8,9} and coronary microvessels.¹⁰

In UA, a coronary microvessel dysfunction may also be facilitated by the intermittent ischemia that in experimental and human studies has been found to elicit an accelerated inflammatory response by cardiac tissues.^{11,12} Several functional studies have described a reduced capacity for vasodilation of small coronary vessels in anginal patients¹³ and an improper regulation of coronary microcirculation to the enhanced oxygen demand.^{14,15} A recent study has shown that the expression levels of both heat shock protein 72 and endothelial nitric oxide synthase are higher in biopsy specimens from the right atrium of UA patients than of patients with stable angina (SA),¹⁶ raising the possibility that the myocardium may be involved in UA. However, no information is available on whether the myocardium and coronary microvessels are involved in the inflammatory reaction associated with UA. Therefore, we planned the present study with the following objectives: (1) to investigate whether the myocardium is involved in UA, and if so, (2) to identify the cellular and molecular features of the myocardial inflammation and (3) to examine the mechanism(s) responsible for myocardium involvement.

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Characteristics of Controls and Anginal Patients

Characteristics	Controls (n=15)	Stable Angina (n=12)	Unstable Angina (n=24)
Males/Females	11/4	10/2	18/6
Age, y	54±12	62±7	59±12
Weight, kg	77.5±7.2	78.5±7.4	75.3±8.4
Smoking, %	9 (60)	8 (67)	17 (70)
Cholesterolemia, mmol/L	4.3±0.9	5.3±0.7	5.4±0.5
Diabetes, %	2 (16)	3 (25)*	6 (25)*
Hypertension, %	6 (40)	5 (42)	11 (46)
Left ventricular mass, g/m ²	112±14	111±12	113±14
LVEF, %	60.4±5.2	53.6±4.5	54.9±5.1
Mean aortic pressure, mm Hg	95.1±11.9	98.2±12.6	100.7±12.6
Mean angiographic score†	...	15.8±4.3	16.2±4.2
Coronary blood flow, mL/min	85.1±8.4	80.6±4.5	82.6±13.8
CVR, mm Hg/mL per min	1.26±0.34	1.52±0.49	1.58±0.53

LVEF indicates left ventricular ejection fraction; CVR, coronary vascular resistance. Values are mean±SD or number (percentage).

* $P<0.01$ vs controls.

†According to the American Heart Association.

Materials and Methods**Study Population**

Twenty-four patients with UA in class IIB (n=10) and IIIB (n=14) of Braunwald's classification and 12 with SA in Canadian Class II (n=7) and III (n=5) were investigated. Patients with UA and SA were considered for inclusion in the study if they had a 2-vessel disease, one of which was the left anterior descending (LAD) artery, and they had been scheduled for a coronary artery bypass graft (CABG) because unsuitable for percutaneous transluminal angioplasty. More precisely, 7 patients had disease of the LAD and circumflex artery (LCx), 2 of the LAD and obtuse marginal artery (OMA), and 15 patients of the LAD and coronary right artery (CRA). The distribution of coronary vessel disease and baseline clinical characteristics of the study population are reported in the Table. Diagnosis and risk evaluation of UA and SA patients were performed according to the ACC/AHA guidelines.^{17,18} High-risk patients and patients with recent (ie, within the preceding 3 months) myocardial infarction were not accepted for the study. Only patients with recent onset (<7 days before admission) of angina who had at least 2 episodes of angina at rest or 1 episode lasting more than 20 minutes during the preceding 24 hours associated with transient ischemic ST-segment changes and troponin T levels <0.1 ng/mL (troponin T-negative) were included in the study. UA patients received aspirin (100 mg/d) and nitrates. In addition to this therapy, 15 patients were on β -blockers, 17 were treated with heparin, and 8 with calcium-antagonists. Anginal episodes were recorded up to surgery. Patients with SA were on aspirin (100 mg/d) and nitrates. Patients who on admission had had recent infectious diseases, known or suspected neoplasms, erythrocyte sedimentation rate >20 mm/h were not considered eligible for the study. The protocol of this study complies with the principles of the Helsinki declaration and was approved by the Ethical Committee of our Institution. All patients gave their informed consent to participate and to have blood samples and biopsy specimens used for the study.

Experimental Procedures

Patients with the above mentioned characteristics were enrolled in the study after coronary angiography, which was performed from 48 to 96 hours after admission. Severity of coronary lesions and presence and grade of coronary collateral circulation were evaluated. No patients had ongoing anginal symptoms when

undergoing surgery. CABG was performed from 6 to 9 days after coronary angiography. On the day preceding surgery, 10 mL of venous blood was withdrawn from UA and SA patients as well as from 15 healthy controls to measure serum concentration of sIL-2R, a long-lasting marker of lymphocyte activation.¹⁹ Immediately after sternotomy and before inducing cardioplegia, 2 transmural biopsies (10×0.5 mm) were taken from the anterolateral wall of the left ventricle close to the apex in the distribution territory of the LAD artery through a biopsy needle (MN1416, diameter 2.1 mm; BIP GmbH). Because this area was potentially ischemic in all the patients, in 9 of the 15 with coronary disease involving the LAD and CRA, an additional biopsy was taken from the free left ventricle wall in an area which appeared normally perfused at ²¹¹Thallium scintigraphy performed before coronary angiography and was supplied by obtuse marginal branches angiographically free from atherosclerotic lesions. The surgeon did not take the biopsy specimens if the area selected for biopsies appeared as a site of an old infarction. The amount of tissue obtained was sufficient to allow quantification and localization of mRNAs and immunohistochemistry studies of the corresponding proteins for each patient. Cardiac specimens were also obtained from the anterolateral wall of the left ventricle of 6 patients with mitral stenosis who underwent surgical valve replacement (control hearts, CHs). Handling of biopsy specimens for RT-PCR, hybridization in situ, and immunohistochemical studies were performed as previously described²⁰ (see expanded Materials and Methods section presented in the online data supplement available at <http://www.circresaha.org>).

Immunohistochemical Detection of Inflammatory Cells and Morphometric Analysis

The presence and identification of activated inflammatory cells in the myocardium were determined by immunohistochemical analysis for major histocompatibility class II molecules (HLA-DR) on adjacent serial sections stained for macrophages, T lymphocytes, and endothelial cells using human monoclonal antibodies (see detailed list in expanded Materials and Methods). The presence of neutrophils was also investigated by immunostaining for elastase. Immunostains were visualized through the avidin-biotin horseradish peroxidase visualization system (Vector Laboratories). The number of DR⁺ inflammatory (lymphocytes and macrophages) and endothelial cells was expressed as cells/mm². Collagen staining was expressed as the percentage of Picrosirius red area to the total area of the biopsied sample.¹² The quantitative analysis was performed by means of Zeiss Image Software. The entire stained biopsied sample was scanned with Adobe Photoshop software (Adobe Systems) and a Leaf Microluminal digital camera.

RT-PCR Quantification of mRNA for Cytokines, MCP-1, and iNOS and In Situ Hybridization Studies

mRNAs for interleukin (IL)-1 β , tumor necrosis factor- α (TNF- α), IL-6, interferon- γ (IFN- γ), monocyte chemoattractant protein-1 (MCP-1), and inducible nitric oxide (iNOS) were quantified by RT-PCR, as previously reported,²⁰ by means of specific primers and expressed as ratios to the constitutively expressed gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described elsewhere.²¹ Characteristics of primers and operative conditions for RT-PCR are reported in the expanded Material and Methods. The in situ hybridization procedure was performed with specific cDNA photobiotin-labeled (Vector) probes.^{20,21} The localization of cytokine and iNOS genes was done on serial sections adjacent to those immunostained for macrophages, T lymphocytes, and endothelial cells (see online data supplement).

Immunohistochemical Analysis for Cytokines and iNOS Proteins

Cytokine proteins were assayed by immunohistochemical analysis according to the avidin-biotin peroxidase method using primary monoclonal antibodies against human TNF- α (Santa Cruz Biotech-

nology), IL-6 (RD System), IFN- γ (Sigma), and iNOS (Transduction Laboratory).

Statistical Analysis

Data are expressed as mean \pm SD. Serum concentrations (median values) of sIL-2R are given as medians and range. Groups were compared by Student's *t* test for paired and unpaired data. We used one-way analysis of variance (ANOVA), followed by Tukey's multiple-range comparison test, when appropriate, to analyze the differences among the 3 groups. For sIL-2R, we applied Kruskal-Wallis's nonparametric test for one-way ANOVA (H test), followed by the post hoc test to examine the differences among the 3 groups. We used linear regression analysis to test the relationships between severity of angina and (1) serum sIL-2R concentration and (2) the expression levels of cytokine and iNOS genes. We also obtained correlations between the severity of coronary lesions and each of these variables. Statistical significance was taken as $P < 0.05$. We used BMDP statistical software (BMDP Statistical Software Inc) for all calculations.

Results

Clinical Course

No patients had heart failure, myocardial infarction, or died before, during, or after the surgical procedure. Nine out of 24 UA patients and 2 out of 12 SA patients suffered anginal episodes in the interval between enrollment and surgery: more precisely, 1 patient had 1 episode, 5 had 2 episodes, and 3 patients had from 3 to 5 episodes. No anginal episode lasted more than 5 to 6 minutes and only 2 patients had angina in the 2 days before surgery.

Serum concentrations (median values) of sIL-2R were significantly ($P < 0.001$) higher in UA (147 pmol/L, range 93 to 212.5) than in SA patients (91 pmol/L, range 73.2 to 117.2) and controls (83 pmol/L, range 71 to 112.7). Twenty-two out of 24 UA patients (87%) and 1 of 12 SA patients had sIL-2R values above the confidence limits of controls. No relationship was found between serum levels of sIL-2R and either the severity of angiographic coronary lesions or the severity of angina in terms of number and duration of the anginal episodes ($r = 0.12$, $P = 0.73$; $r = 0.16$, $P = 0.71$; and $r = 0.15$, $P = 0.68$; respectively).

Collateral blood flow was angiographically demonstrable in only 7 patients (grade 0 to 3) and in 4 patients supplied the area of ventricular wall from which biopsy specimens were taken. The expression levels of cytokine and iNOS genes did not differ between these patients and those without collateral blood flow.

Inflammatory Cells in Myocardium and Morphometry

Biopsy specimens from UA patients showed numerous cells expressing DR molecules, which, conversely, were very rare in SA patients and absent in CHs (Figure 1). In adjacent serial sections, the great majority of DR⁺ cells was represented by endothelial cells identified by both CD31 and von Willebrand immunostaining, and the remaining DR⁺ cells were identified as CD68⁺ (macrophages) or CD3⁺ (lymphocytes) (Figure 2). DR⁺ lymphocytes and macrophages were exclusively localized in myocardial interstitium. The quantitative distribution of the DR⁺ cells in the biopsies from UA, SA, and CHs and in potentially ischemic and nonischemic left ventricular areas is reported in Figure 3. No elastase positive cells (neutrophil

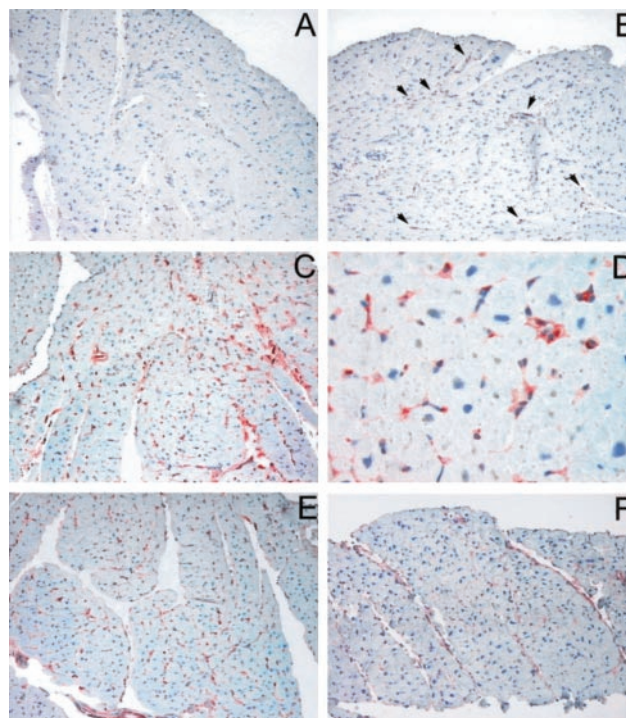


Figure 1. Immunostaining for cells expressing DR antigens in myocardial biopsies. Positive signal revealed by red-brown staining. DR⁺ cells are undetectable in CHs (A) and very few in SA specimens (B), whereas numerous DR⁺ cells are present in UA biopsy specimens (C and D) without significant differences between ischemic (E) and nonischemic areas (F). A, B, C, E, and F, magnification $\times 100$; D, magnification $\times 400$. Signal was revealed by avidin-biotin peroxidase system. Arrows indicate DR⁺ cells in B.

leukocytes) were detectable in any of the biopsies. Collagen content was less than 3% in UA biopsies, whereas it ranged between 4.3% and 8.6% in SA biopsies.

Expression Levels of Cytokine, MCP-1, and iNOS mRNAs

RT-PCR showed that the expression levels of mRNAs were negligible or undetectable in biopsy specimens from CHs (Figure 4). In SA biopsies, mRNAs for IL-1 β , IL-6, IFN- γ , and MCP-1 were undetectable or negligible, whereas only TNF- α and iNOS genes were clearly detectable and expressed at a higher level than in CHs ($P < 0.05$ and 0.01, respectively) (Figure 4). By contrast, in UA biopsies the genes for TNF- α , IL-6, IFN- γ , and iNOS were overexpressed, from 2.4-fold for TNF- α ($P < 0.001$) to 6.1-fold for IL-6 ($P < 0.001$) when compared with SA hearts (Figure 4). IL-1 β and MCP-1 gene expression was undetectable or negligible. In patients with LAD plus CRA disease, the expression levels of mRNAs for cytokines and iNOS were not significantly different for potentially ischemic and nonischemic areas (Figure 4).

Correlation Between Severity of Microvessel Inflammation and Clinical and Systemic Variables

The degree of the microvessel inflammatory response computed for each patient as the sum of expression levels of TNF- α , IL-6, IFN- γ , and iNOS genes was not correlated with

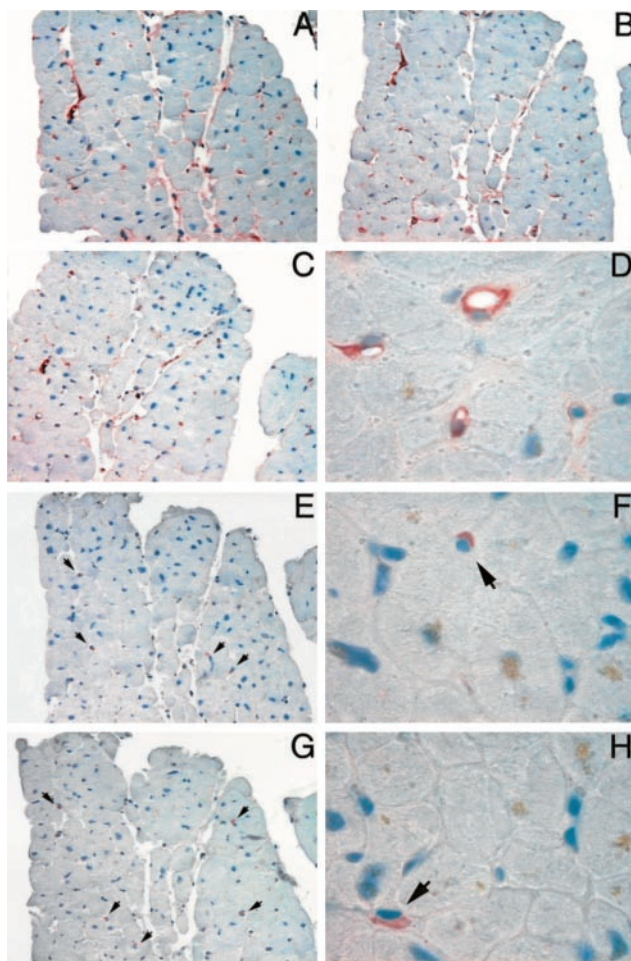


Figure 2. Immunohistochemical staining for HLA-DR⁺ cells (A), CD31⁺ (B), and von Willebrand⁺ (C and D) endothelial cells, CD3⁺ T lymphocytes (E and F), and CD68⁺ macrophages (G and H) in adjacent serial sections of myocardial biopsies from UA patients. Positive signal revealed by red-brown staining (A, B, C, E, and G, magnification $\times 200$; D, F, and H, magnification $\times 1000$). Arrows indicate lymphocytes in E and F and macrophages in G and H.

either the severity of coronary lesions ($r=0.19$, $P=0.85$) or the severity of angina defined as number or as duration of anginal episodes ($r=0.20$, $P=0.87$ and $r=0.21$, $P=0.89$, respectively). Conversely, the degree of the myocardial inflammatory response was positively correlated with plasma levels of sIL-2R ($r=0.43$, $P=0.05$).

Localization of Cytokine and iNOS mRNAs

Negative and positive controls for in situ hybridization showed that the signal was specific for mRNA, and that mRNA in the biopsies was intact. In CHs, mRNAs for cytokines and iNOS were undetectable. In SA hearts, gene signals for IFN- γ and IL-6 genes were undetectable, whereas signals for TNF- α and iNOS mRNAs were weakly expressed (Figure 5). Conversely, in UA biopsy specimens, the genes for all the cytokines examined and iNOS were detectable (Figure 5) both in potentially ischemic and nonischemic areas (online Figure 1 in the online data supplement available at <http://www.circresaha.org>). TNF- α , IL-6, and iNOS genes

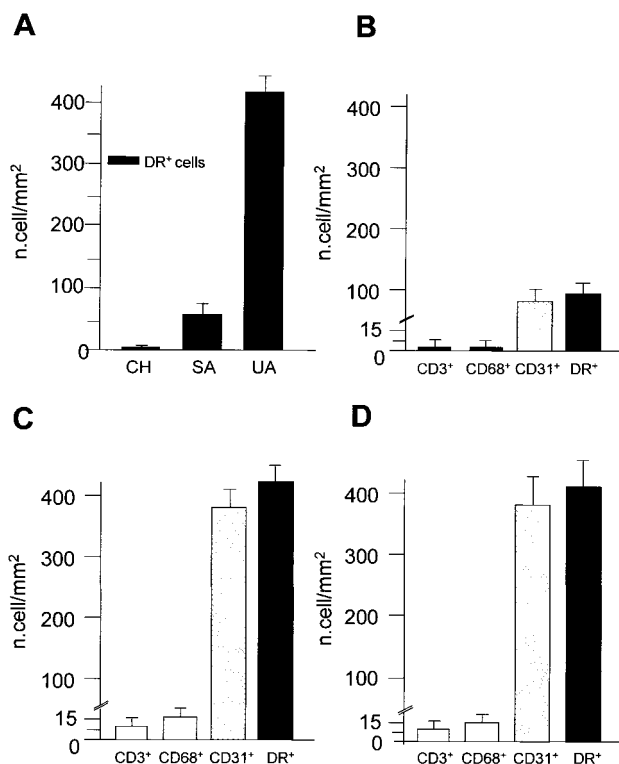


Figure 3. A, Bar graphs show mean number \pm SD of all DR⁺ cells in control hearts (CHs), SA, and UA hearts. Bar graphs show mean number \pm SD of all DR⁺ cells, T lymphocytes (CD3⁺), macrophages (CD68⁺), and endothelial cells (CD31⁺) in biopsies from SA hearts (B) and UA hearts examined in potentially ischemic (C) and nonischemic (D) myocardial areas.

were mainly expressed on endothelial cells and more weakly on interstitial cells. The IFN- γ gene was detectable both in endothelial cells and lymphocytes, whereas TNF- α , IL-6, and iNOS genes were also expressed on macrophages (Figure 6). Cardiomyocytes did not express mRNA either for cytokines or iNOS (Figure 6).

Immunohistochemical Localization of Cytokine and iNOS Proteins

We did not find immunoreactivity for cytokine and iNOS proteins in CHs, and only weak signals for TNF- α and iNOS proteins in SA biopsies (Figure 7). Conversely, in UA hearts, immunoreactivity for TNF- α , IL-6, IFN- γ , and iNOS proteins was highly expressed and predominantly localized in microvascular endothelial cells, in lymphocytes and macrophages but not in cardiomyocytes (Figure 7).

Discussion

Our results showed that an acute inflammatory process involving coronary microvessels, but not cardiomyocytes, is detectable in UA patients. The microvessel inflammation is revealed by the presence of inflammatory (lymphocytes and macrophages) and resident cells, particularly endothelial cells, expressing HLA-DR molecules, as well as the increased mRNA levels for several cytokines (TNF- α , IL-6, and IFN- γ) and iNOS in the left ventricle biopsies from UA. Although RT-PCR allows only a semiquantitative assay, the marked

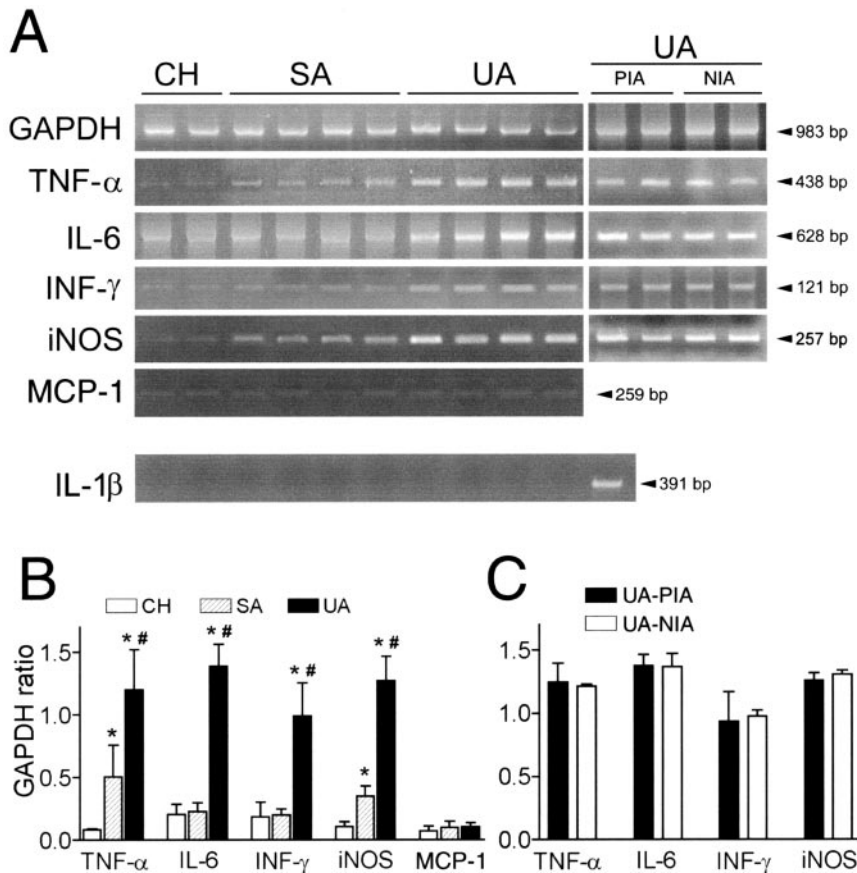


Figure 4. A, RT-PCR expression of mRNAs for GAPDH, TNF- α , IL-6, INF- γ , iNOS, MCP-1, and lack of IL-1 β gene expression in biopsy specimens from control, SA, and UA hearts. Cytokine and iNOS mRNA levels in potentially ischemic and nonischemic areas of UA hearts are separately shown on the right (PIA, potentially ischemic areas; NIA, non-ischemic areas). The positive control for IL-1 β was obtained using human lymphocytes. Representative experiments are shown. B, Bar graphs show densitometric quantification of RT-PCR products of cytokines and iNOS obtained in control, SA, and UA hearts. C, Densitometric quantification of RT-PCR products of cytokines and iNOS in potentially ischemic and nonischemic areas of the same UA patients. Data are presented as mean \pm SD. * P < 0.01 vs control and # P < 0.01 vs SA specimens.

differences in the expression levels (from 2.4- to 6.1-fold) of cytokine and iNOS genes between UA and SA patients and even more versus CHs by far overcome the limits of the method and corroborate the existence of an inflammatory process. In situ hybridization studies of cytokine and iNOS genes and immunostaining of their proteins revealed that the inflammatory process was essentially confined to endothelial cells and, to a lesser extent, to interstitial cells.

Because myocardial ischemia or necrosis may induce an acute inflammatory reaction, it is crucial to analyze the meaning of the inflammatory process found in UA patients. According to our enrollment criteria, UA patients were troponin-negative, and myocardial biopsies were taken before extracorporeal circulation, so the inflammatory process cannot be considered a reaction to myocardial necrosis²² or surgery.²³ Myocardial inflammatory reaction may also be induced by an acute ischemic episode followed by reperfusion according to the classic ischemia/reperfusion injury.²⁴ In this situation, polymorphonuclear leukocytes, mainly neutrophil leukocytes, are recruited.²⁴ Immunostaining of myocardial biopsies from our patients did not show elastase, the marker of neutrophils recruitment. A recent study reported that neutrophils undergo activation during the passage across the coronary vascular bed in UA patients.²⁵ Myocardial ischemia alone did not suffice to induce the inflammatory state of the coronary bed in UA patients, because in the same study, neutrophils were not activated in patients who had multiple episodes of variant angina.²⁵ In our UA patients, intermittent ischemia could have induced myocardial inflam-

matory process like that observed in the late phase of ischemic preconditioning¹¹ or hibernating myocardium.²⁶ In UA patients with LAD and CRA disease and insignificant lesions of the LCx artery, the number of DR⁺ cells and the expression levels of cytokines and iNOS genes were not different between the potentially ischemic and nonischemic areas, that is, areas normally perfused at ²¹¹thallium scintigraphy. Moreover, several specific histological and molecular features differentiate coronary microvessel inflammation found in UA patients from the hibernating myocardium and the late phase of ischemic preconditioning. Hibernating myocardium is associated with degeneration of cardiomyocytes, loss of contractile material²⁷ fibrosis and MCP-1 gene overexpression.¹² By contrast, all these findings were not observable in the UA coronary microvessel inflammation. In the late phase of preconditioning, IL-1 β and TNF- α genes are coexpressed²⁸ and the iNOS gene is selectively upregulated in the myocytes, but not in the small vessels and fibroblasts.^{11,29} In UA inflammation, instead, IL-1 β was not expressed and iNOS mRNA was upregulated in the microvessels but not in the myocytes, thus indicating that myocytes were not involved in the coronary microvessel inflammation. C-reactive protein (CRP) at 5 μ g/mL exerts a direct inflammatory effect on both cultured human umbilical vein and coronary artery endothelial cells.³⁰ Because we did not know the CRP intracoronary levels of our patients, we cannot rule out that CRP contributed to the endothelial inflammatory process. However, the high number of DR⁺ endothelial cells strongly suggests an immunological activation³¹ independently of any

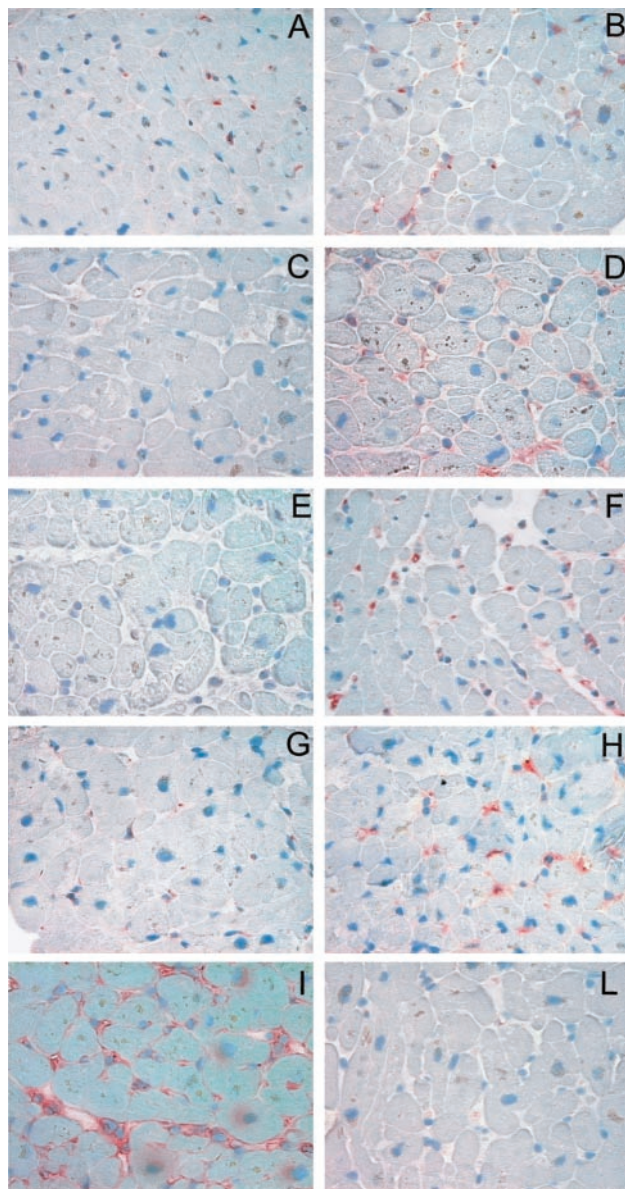


Figure 5. In situ hybridization for TNF- α (A and B), IL-6 (C and D), IFN- γ (E and F), iNOS (G and H), GAPDH (I), and plasmid vector pBR322 (L) in left ventricular biopsies from SA (A, C, E, and G) and UA specimens (B, D, F, and H). Positive mRNA signal was revealed by red-brown staining. In SA hearts, positive mRNA signal was almost absent, whereas in UA hearts, mRNAs for cytokines and iNOS were expressed in interstitial and, especially, in endothelial cells. Cardiomyocytes did not express mRNA either for cytokines or iNOS. Magnification $\times 400$.

possible inflammatory action by CRP. The different histological and molecular characteristics of the coronary microvessel inflammation observed in our patients and the ischemia-related inflammatory reactions, as well as the lack of significant differences in the expression levels of inflammatory cytokine and iNOS genes between the nonischemic and potentially ischemic myocardial areas, make it highly unlikely that the coronary microvessel inflammation is a direct consequence of intermittent ischemia. We cannot exclude, however, that the microvessel inflammation may be an indirect consequence of transiently increased sympathetic

activity³² or an ischemia-related stress response and, therefore, a process extending beyond the directly ischemic region. Nor can we rule out that subclinical showers of microemboli or active material released by multiple active plaques³³ might have reached the myocardial areas not subjected to ischemia.

Immune Reaction

The major distinctive feature of coronary microvessel inflammation was the expression of HLA-DR molecules on the inflammatory cells (T cells and macrophages) and endothelial cells. Expression of DR molecules provides evidence that antigen presentation occurred and an immunological reaction is ongoing or has recently happened.³⁴ This contention is strengthened by the expression of IFN- γ gene and protein both in lymphocytes and endothelial cells and by the simultaneous increase in sIL-2R in serum, a marker of recent T-cell activation.¹⁹ The expression of the IFN- γ gene and protein together with DR molecules on the endothelial cells indicates

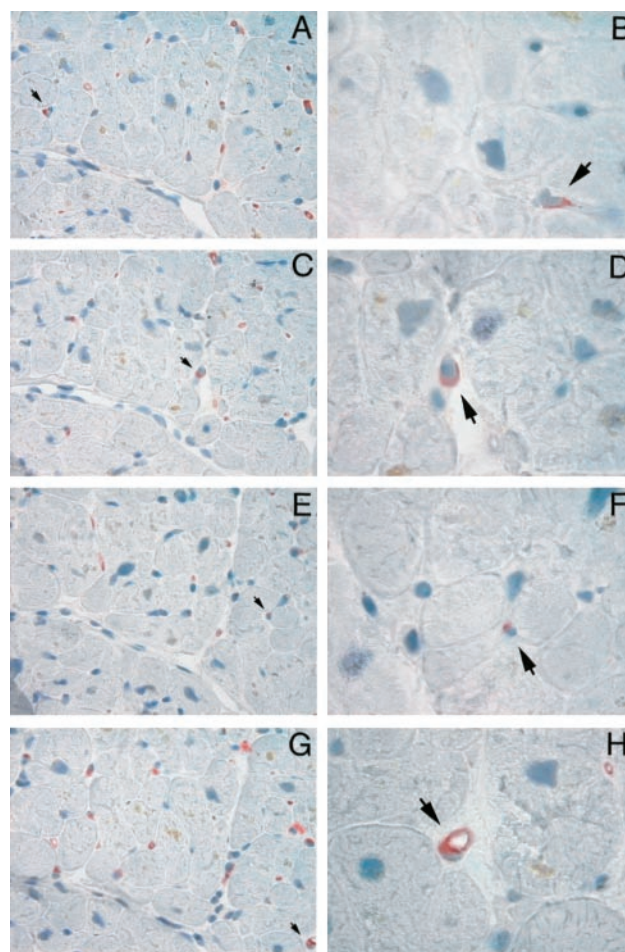


Figure 6. In situ hybridization for TNF- α (A), IL-6 (C), IFN- γ (E), and iNOS (G) and their localization in different cellular types in myocardial biopsies from nonischemic areas of UA patients. Serial adjacent sections were immunostained for macrophages (CD68⁺) (B and D), T lymphocytes (CD3⁺) (F), and endothelial cells (CD31⁺) (H) for the localization of cytokine and iNOS genes on different cell types. A, C, E, and G, magnification $\times 400$; B, D, F, and H, magnification $\times 1000$. Arrows indicate macrophages in A, B, C, and D, lymphocytes in E and F, and endothelial cells in G and H.

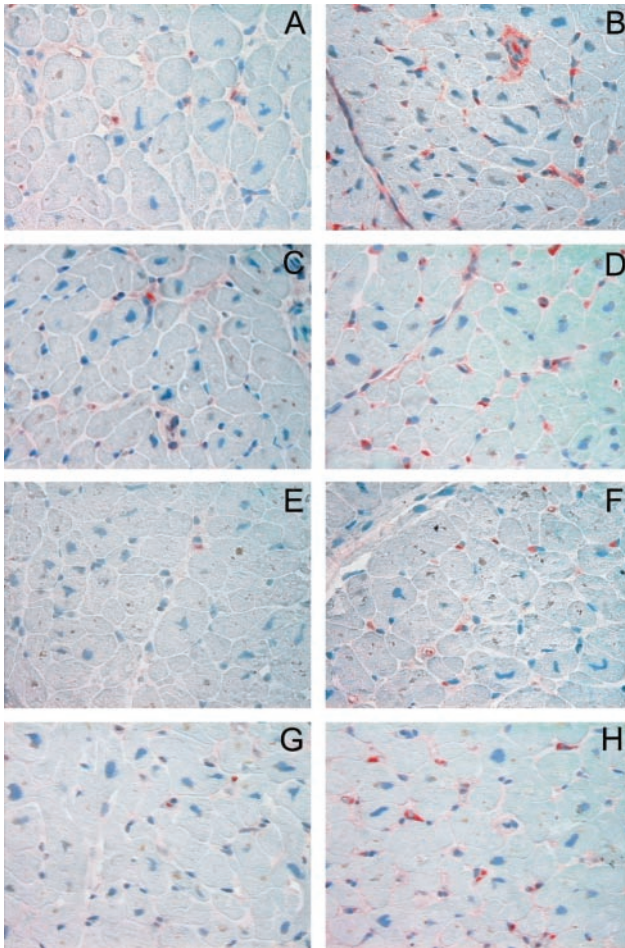


Figure 7. Immunostaining for TNF- α (A and B), IL-6 (C and D), IFN- γ (E and F), and iNOS (G and H) proteins in SA (A, C, E, and G) and UA (B, D, F, and H) myocardial biopsy specimens. Weak signals for cytokines and iNOS were detectable in SA specimens, whereas strong signals were evident on interstitial and, especially, on endothelial cells in UA biopsies specimens. No signals are detectable on myocytes. Magnification $\times 400$.

the participation of the microvascular endothelium in the immune reaction.³⁵ These findings are consistent with histological data pointing to the occurrence of a recent immune activation within the culprit plaques of UA patients. These plaques are characterized by a markedly higher number of T cells, macrophages, and smooth muscle cells expressing DR molecules than that found in SA patients.^{36–38} Moreover, T cells of culprit plaques express IL-2R on their membrane,³⁷ suggesting a recent immune activation.³⁹ Human and experimental studies have shown that antigens are formed into the plaques during LDL oxidation or degradation.⁴⁰ T-cell clones from human atherosclerotic plaques respond to oxidized LDL by proliferation and IFN- γ secretion,⁴¹ and circulating T cells from UA patients, but not from SA patients or normal control subjects, proliferate in vitro in response to oxidized LDL and/or to autologous proteins obtained from coronary culprit lesions.⁴²

Microvessel Inflammation and Angina

We found a diffuse inflammatory process with major distinctive features of immune reactions in UA patients. Patients

were on full medical therapy, and the interval between their admission and obtaining biopsies (from 9 to 20 days) did not permit us to investigate the relationship between the microvessel inflammation and the clinical data. Only 37% of the patients experienced few, moderate anginal episodes during the study period. Thus, the clinical significance of the microvessel inflammation remains to be defined. It appears reasonable to suppose that a widespread coronary microvessel inflammation might contribute to the paradoxical increase in the microvessel resistance during ischemia in UA patients¹⁵ or to the alterations in coronary flow in myocardial territory perfused by arteries without stenosis or culprit lesions in patients with UA or recent infarction.^{13,43} However, we are aware that further studies are needed to explore the significance of the microvessel inflammation. Conversely, the results of the present study have a pathophysiological meaning because they indicate that in UA the inflammation is not confined to the culprit unstable plaque but involves coronary microvessels as well. Our results are consistent with recent angiographic, angioscopic, and intracoronary ultrasound studies that show that, in addition to the responsible lesion, patients with UA or myocardial infarction may harbor multiple plaques, eroded and thrombosed, or with the characteristics of the vulnerable plaques diffused on the 3 coronary arteries.^{44–46} Finally, the distinctive features of immunomediated inflammation both in unstable plaques and coronary microvessels suggest that immune factors may play a role in precipitating the acute activation of the chronic smoldering inflammatory process of coronary atherosclerosis.

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Title of Manuscript:
Immuno-mediated and ischemia-independent inflammation of coronary microvessels in unstable angina.

Online Supporting Data:
Expanded materials and methods section

Transmural left ventricle biopsies and tissue processing

Immediately after sternotomy and before inducing cardioplegia, transmural biopsies (10 x 0.5 mm) were taken through a biopsy needle (MN1416 diameter 2.1 mm; BIP GmbH). One of the biopsies was collected in sterile RNAase-free tubes immediately frozen in liquid nitrogen in the operating theater and thereafter transferred to -80°C for storage. The other one was used for immunohistochemical studies. This sample was immediately immersed in buffered 10% formalin for 18 hours, dehydrated in an alcohol series (50%, 75%, 95% ethanol and absolute) and, then, in xylene and finally embedded in a paraffin block.

Immunohistochemistry

Paraffin blocks obtained from bioptic specimens were serially cut into sequential 3-5 µm sections by microtomy. At least 25 serial adjacent sections from 1 biopsy from each patient were mounted for immunohistochemistry and stained at 3 cutting levels with hematoxylin and eosin for morphologic evaluation. Serial sections were deparaffinized with xylene (3 times for 5 min) and immediately treated with 3% hydrogen peroxide (10 min) to block endogenous peroxidase. After washing in phosphate buffered saline (PBS) (3 times for 5 min), non specific binding was blocked with normal blocking serum and then the sections were incubated overnight at 4°C with the primary antibody. All antibodies were diluted in PBS containing 10% normal blocking serum. After washing (3 times for 10 min) with PBS, the sections were incubated at room temperature for 1 hour with biotinylated secondary antibody. After an extensive wash with PBS (3 times for 10 min), antibody binding was revealed with the avidin-biotin peroxidase complex technique (Vector Laboratories) and 3-amino-9-ethylcarbazole was used as the chromogenic substrate. Cells nuclei were counterstained with hematoxylin. Negative controls were obtained by omitting the primary antibody.

Immunohistochemical detection of inflammatory cells

The presence and identification of activated inflammatory cells in the myocardium were determined by immunostaining for major histocompatibility class II antigen (HLA-DR) on sections adjacent to serial sections stained for macrophages, T lymphocytes and endothelial cells. The presence of neutrophils was also investigated by immunostaining for elastase.

The following primary antibodies were used: mouse monoclonal antibody anti human HLA-DR (Dako, 1:50 dilution), mouse monoclonal antibody anti macrophages CD68 (Dako, 1:50 dilution), mouse monoclonal antibody anti T lymphocytes CD3 (Santa Cruz Biotechnology, 1:30 dilution), mouse monoclonal antibody anti endothelial cells CD31 (Dako, 1:30 dilution) and von Willebrand (Sigma, 1:120 dilution), and mouse monoclonal antibody anti neutrophil elastase (Dako, 1:40 dilution).

Immunohistochemical analysis for cytokines and iNOS.

Cytokine proteins were assayed by immunohistochemical analysis according to the avidin-biotin peroxidase method. The following primary antibodies were used: mouse monoclonal antibodies against human INF- γ (Sigma, 1:50 dilution), IL-6 (RD System, 1:400 dilution), TNF- α (Santa Cruz Biotechnology, 1:50 dilution) and against iNOS (Transduction Laboratory, 1:50 dilution).

Quantitative analysis was performed using Zeiss Image software. The entire stained biopsied sample was scanned using Adobe Photoshop software (Adobe Systems) and a Leaf Microlumina digital camera. Collagen staining was expressed as the percentage of the picrosirius-red stained area to the total area of the segment. The number of DR positive (DR⁺) inflammatory (lymphocytes and macrophages) and endothelial cells was expressed as cells/mm².

Reverse transcriptase-polymerase chain reaction (RT-PCR) quantification of TNF- α , IL-6, INF- γ , IL-1 β and iNOS mRNA levels

Total mRNA was isolated from homogenized frozen samples using TRIzol reagent (BRL-Life Technologies), as outlined by the manufacturer, and reverse transcribed using oligo dT-20. Levels of TNF- α , IL-6, INF- γ , IL-1 β and iNOS gene expression were quantified by RT-PCR as previously described (1). To ensure that different amounts of RT-PCR products on myocardial biopsies were not due to different starting concentrations of mRNA, RT-PCR analysis was performed for the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA on serial 2-fold dilutions of cDNA for each sample. The last dilution giving a positive reaction for GAPDH was used to equalize the amount of cDNA used in each RT-PCR. The sequences of the primers used and RT-PCR

conditions are showed in Table 1. All primers were purchased from Pharmacia. The RT-PCR products were electrophoresed in a 2% agarose gel and visualized with ethidium bromide by UV light transillumination. Band density was analysed using a computerized image densitometer (Qwin, Leica) and the expression level for each target gene was calculated as the ratio of the density of the target gene band versus that of the GAPDH band (densitometric ratio [mRNA for TNF- α , IL-6, INF- γ , IL-1 β and iNOS / mRNA for GAPDH]).

The variability of the reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was assayed by measuring band densities obtained for each target gene for 5 samples assayed five times in the same amplification and again in four different amplifications. The intra-assay and inter-assay variation coefficients were $4.1 \pm 1.2\%$ and $4.8 \pm 1.4\%$, respectively.

Localization of TNF- α , IL-6, INF- γ and iNOS mRNAs in the myocardium by in situ hybridization

The in situ hybridization procedure was performed using complementary cDNA photobiotin labelled probes for GAPDH (pHcGAP, ATCC n.57090), TNF- α (pE4, ATCC n.39894), IL-6 (PT7T3D, ATCC n.3127523), INF- γ (p52, ATCC n.65948) and iNOS (ATCC n.1351820) purchased by Vector Laboratories as previously described in details (1). The biotinilated probe was mixed in 60 μ L hybridization buffer, applied to each section, and hybridizations were performed at 55°C overnight in humidified chamber. Washes included stringent wash in 1x SSC for 30 minutes and 0.1x SSC for 1 hour at 55°C. The streptavidin-biotinylated horseradish peroxidase complex in buffered sodium chloride was used as detection reagent and 3-amino-9 ethyl-carbazole (AEC, Sigma) as cromogenic substrate for 5 min at room temperature. The cells nuclei were counterstained with hematoxylin (Mayer's haemalum).

Negative controls were performed by testing the sections with hybridization mixture 1) without the probe, 2) after incubation with RNAase A (0.05 mg/mL = 4.7 Kunitz units/mL) for 1 hour at 37°C, and 3) with application of inappropriate probe (plasmid vector pBR322). Positive controls were obtained for each sample using a cDNA probe for the constitutively expressed gene for GAPDH to ensure that mRNA in myocardial biopsies was intact.

Each stained histological section was examined under a microscope (DMRB, Leica) connected to a computerized image-analysis system (Qwin, Leica) at 400X magnifications.

Legend of Figure 1 of the online supplementary data

Figure 1. In situ hybridization for TNF- α (A,B), IL-6 (C,D), IFN- γ (E,F) and iNOS (G,H) in myocardial biopsies from potentially ischemic (A,C,E,G) and non-ischemic areas (B,D,F,H) of the same UA patients. Cytokine and iNOS genes were mainly expressed on endothelial cells and more weakly on interstitial cells, without differences between potentially ischemic and non-ischemic areas. Cardiomyocytes never express mRNA either for cytokines or iNOS. (magnification x400).

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Online data supplements

Table 1 .Primer sequences and PCR conditions

Target	Primer sequence	Product size	Annealing temperature (°C)	Cycles (n)
GAPDH				
Forward	5'-TGAAGGTCGGAGTCAACGGATTTGGT-3'	983pb	63	32
Reverse	5'-CATGTGGGCCATGAGGTCCACCAC-3'			
TNF α (2)				
Forward	5'-GCCTGTAGCCCATGTTGTAG-3'	438bp	57	30
Reverse	5'-AATGATCCCAAAGTAGACCTGCCC-3'			
IL6 (3)				
Forward	5'-ATGAACTCCTTCTCCACAAGCGC-3'	628bp	65	32
Reverse	5'-GAAGAGCCCTCAGGCTGGACTG-3'			
INF γ (4)				
Forward	5'-GGACCCATATGTAAAAGAAGCAGA-3'	121bp	55	35
Reverse	5'-TGTCACCTCTCCTCTTTCCAATTCT-3'			
IL-1 β (5)				
Forward	5'-AGTACCTGAGCTCGGCAGTGAA-3'	391bp	55	35
Reverse	5'-TACGATCACTGAACTGCAGCC-3'			
INOS (2)				
Forward	5'-CGGTGCTGTATTTCCCTTACGAGGCGAAGAAGG-3'	391bp	62	35
Reverse	5'-GGTGCTACTTGTTAGGAGGTAAGTAAAGGGC-3'			
MCP-1 (6)				
Forward	5'-TTGTGTGCCTGCTGCTCATA-3'	259bp	55	35
Reverse	5'-GGTTTGCTTGTCAGGTGGT-3'			

